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Arrhythmogenic calmodulin E105A mutation alters cardiac RyR2 regulation leading to cardiac dysfunction in zebrafish

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Abstract

Calmodulin (CaM) is a universal calcium (Ca^{2+})-binding messenger that regulates many vital cellular events. In cardiac muscle, CaM associates with ryanodine receptor 2 (RyR2) and regulates excitation-contraction coupling. Mutations in human *CALM1*, *CALM2*, and *CALM3* genes have been associated with life-threatening heart disorders, such as long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia. A novel *de novo* LQTS-associated missense CaM mutation (E105A) was recently identified in a 6-year-old boy, who experienced an aborted first-episode of cardiac arrest. Herein, we report the first molecular characterization of CaM E105A mutation. Expression of CaM E105A mutant in zebrafish embryos resulted in cardiac arrhythmia and increased heart rate, suggestive to ventricular tachycardia. *In vitro* biophysical and biochemical analysis revealed that E105A confers a deleterious effect on protein stability and a reduced Ca^{2+}-binding affinity due to loss of cooperativity. Finally, CaM E105A mutation resulted in a reduced CaM-RyR2 interaction and defective modulation of [^{3}H]ryanodine binding. Our findings suggest that CaM E105A mutation dysregulates normal cardiac function by a complex mechanism involving alterations in both CaM-Ca^{2+} and CaM-RyR2 interactions.

Introduction

Calmodulin (CaM) is a ubiquitous, highly conserved calcium (Ca^{2+})-binding protein that binds and regulates a number of different protein targets, thereby affecting a wide range of vital cellular processes.[1] CaM acts as an intracellular Ca^{2+} sensor, decoding downstream Ca^{2+} signals and by undergoing conformational changes, binds specifically to its multiple protein partners in a Ca^{2+}-dependent manner.[2] CaM is a relatively small protein composed of 148 amino acids with a with very basic domain architecture. Based on its crystal structure, it consists of two globular domains located at the N- and C- termini (N- and C- lobes), separated by a flexible α-helix, in a dumbbell-resembling conformation. Each lobe consists of two EF hand motifs, with each motif binding one Ca^{2+} ion.[3] Thus, the total Ca^{2+} binding capacity of CaM is four Ca^{2+} ions, allowing it to interact with multiple protein targets in both apo-CaM (Ca^{2+}-free CaM) and Ca^{2+}-CaM (Ca^{2+}-loaded CaM) forms. Interestingly, the N- and C-lobes appear to have markedly distinct Ca^{2+} binding characteristics, making the decoding of Ca^{2+} signals a highly complex process.[4, 5]

In cardiac cells, CaM is known to interact and regulate multiple key proteins involved in excitation-contraction coupling (ECC) and Ca^{2+} homeostasis. These proteins include the cardiac ryanodine receptor type 2 (RyR2), voltage operated potassium (K^+), sodium (Na^+) and L-type Ca^{2+} channels.[6] The RyR2 is a large transmembrane high conductance Ca^{2+} release channel that mediates Ca^2 release from the sarcoplasmic reticulum (SR) to activate cardiac muscle contraction.[6] RyR2 gating is crucial for the receptor function and is mediated by a number of modulators (including ions, small molecules and proteins), which regulate the exact timing of channel opening or closing.[7] It is well established that CaM binds to RyR2 stoichiometrically (one CaM per subunit of the homotetrameric RyR2) and inhibits its open probability at both low and high cytosolic Ca^{2+} concentrations. Several studies have proposed that CaM regulation of RyR2 is essential for normal cardiac function.[8-10]

In humans there are three CaM genes (*CALM1, CALM2 and CALM3*), which encode an identical protein and are all expressed in cardiac tissue.[5, 6] A number of recent genetic studies have identified several missense mutations in all three *CALM* genes, in individuals with a family history of severe cardiac disorders and early onset sudden cardiac death.[11-16]

A recent clinical study reported a case of aborted cardiac arrest in a seemingly healthy 6-year-old boy who exhibited profound QT prolongation with an increasing heart rate before the recurrence of polymorphic ventricular tachycardia at a pediatric intensive care unit.[17] The patient had no family history of heart disease, while his parents' ECGs appeared to be normal. Genetic screening revealed a novel de novo missense variation c.A314>C in exon 5 of *CALM1* gene of this patient.[17] This nucleotide change results in the substitution of a conserved glutamic acid (E) with an alanine (A) residue (p.E105A) within the third EF-hand motif in the C-domain of CaM protein.

In the present study, the *in vivo* functional effects of CaM E105A mutation on normal zebrafish embryonic heart function were investigated following human CaM wild type (CaM^{WT}) and CaM E105A mutant (CaM^{E105A}) complementary RNA (cRNA) microinjection experiments in zebrafish embryos. CaM^{WT} and CaM^{E105A} mutant were bacterially expressed and affinity purified as recombinant proteins and their biophysical properties were analyzed by circular dichroism (CD) and thermal denaturation experiments. The Ca^{2+}-binding affinities of both the N- and C-lobes of CaM^{E105A} mutant were determined by monitoring the intrinsic tyrosine and phenylalanine fluorescence and compared to those of CaM^{WT}. Furthermore, co-immunoprecipitation experiments of native RyR2 with CaM^{WT} and CaM^{E105A} mutant were employed to compare their relative RyR2-binding affinities. Finally, the functional effect of CaM E105A mutation on the RyR2 activity was examined with isolated SR membranes via a [^{3}H]ryanodine binding assay. Our findings suggest that the arrhythmogenic CaM E105A mutation dysregulates the normal embryonic heart function in zebrafish by a complex

mechanism, which involves defects in both CaM-Ca^{2+} binding and CaM-RyR2 interaction and regulation.

Material and Methods

A detailed description of methods is provided in the Supplementary Material and Methods section.

Results

Expression of CaM^{E105A} mutant in zebrafish embryos mimics patient's ventricular tachycardia

To investigate any potential *in vivo* functional effects of the LQTS-associated CaM E105A mutation on zebrafish embryonic heart function, we generated and injected synthetic cRNA corresponding to CaM^{WT} and CaM^{E105A} mutant into zebrafish embryos. Injected zebrafish embryos were then raised up to 72 hours post-fertilization (hpf) and their cardiac function was assessed. As shown in Figure 1A (left panel), expression of CaM^{WT} and CaM^{E105A} mutant in zebrafish embryos did not affect normal embryo development, as neither gross morphological changes nor difference in the survival rate were observed in comparison to the control group (uninjected zebrafish embryos). However, we observed a slight change in the heart morphology of CaM^{E105A}-injected zebrafish with ~31.5% embryos exhibiting extended cardiac chambers (Figure 1A, right panel). The average heart rate was significantly increased in this group at an average of 160.5 beats per minute (bpm), (**p = 0.007) when compared to the control group at 152.5 bpm (Figure 1B). Furthermore, cardiac function assessment was performed. Analysis of the cardiac activity of zebrafish ventricle revealed that CaM^{E105A} mutant-injected zebrafish displayed irregular pattern of heart beating in comparison to the median of the CaM^{WT} and control groups (Figure 2). The CaM^{E105A} resulted in increased

arrhythmic potential in this zebrafish model. Collectively, our data suggest that expression of CaM^{E105A} mutant in zebrafish embryos results in cardiac arrhythmia and increased heart rate, mimicking the clinical presentation of the reported 6-year-old boy, which displayed increased heart rate and ventricular tachycardia.

E105A mutation alters CaM thermal stability in the presence of Ca^{2+}

To investigate the effect of E105A mutation on the *in vitro* biophysical and biochemical properties of CaM^{WT} protein, CaM^{E105A} mutant was subcloned into the pHSIE plasmid expression vector and a bacterial expression system was used to express large quantities of recombinant CaM^{WT} and CaM^{E105A} proteins. Expressed recombinant proteins were affinity purified, as we have previously described.[6, 18] Figure 3A shows the affinity-purified untagged CaM^{WT} and CaM^{E105A} recombinant proteins analyzed by SDS-PAGE and immunoblot analysis using an anti-CaM rabbit monoclonal antibody. For each CaM construct, a single protein band with mobility corresponding to the predicted molecular mass (~17.4 kDa) was observed, which was also confirmed by the immunoblot detection.

CD spectra recorded at 4 ^{o}C indicated that CaM^{E105A} has the same overall conformation as CaM^{WT} both in the absence and presence of Ca^{2+} (Fig. 3BI). In the presence of 1mM EDTA, thermal denaturation curves showed only small differences with T_m^1 and T_m^2 ~46 and 63 ^{0}C and ΔH_{vH}^1 and ΔH_{vH}^2 *ca.* -100 and -185 kJ/mol, respectively (Fig. 3BII, III). These values are in good agreement with those previously reported for CaM^{WT}.[19, 20] In the presence of Ca^{2+}, CaM^{E105A} showed a clear bimodal transition with T_m^1 and T_m^2 ~58 and 97 ^{0}C compared to ~90 and 120 ^{0}C observed for CaM^{WT}, respectively. A similar behavior we had observed for the arrhythmogenic CaM^{D130G} mutant in a previous study.[6]

E105A mutation dramatically alters the Ca^{2+}-binding properties of CaM C-domain

Typical normalized fluorescence emission intensity measurements as a function of Ca^{2+} concentration for both CaM^{WT} and CaM^{E105A} are presented in Figures 4A, following changes in the protein N-domain and C-domain respectively. For the C-domain, there is an increase in fluorescence intensity at 320 nm (excitation wavelength: 277 nm) as a function of the free Ca^{2+} concentration in the sample, while for the N-domain the fluorescence intensity decreases at 280 nm (excitation wavelength: 250 nm). Intrinsic calcium-dependent fluorescence intensity changes at these characteristic wavelengths can be used to reliably monitor the occupancy of the EF-hand binding sites in each protein domain.[21] To analyze the data, results from four independent experiments were fitted using global nonlinear regression to a model-independent two-site Adair function and the results are summarized in Table 1. For CaM^{WT} C-domain, binding sites have an apparent K_d almost 3-fold higher than that of the N-domain binding sites (2.97 vs 8.08 μM respectively). The free energy change that accompanies the binding of two Ca^{2+} at the C-domain binding sites was found to be -63.1 kJ/mol with a cooperative free energy change of -9.8 kJ/mol, while the corresponding values for the N-domain binding sites were -58.2 and -4.3 kJ/mol, respectively. These calculations are in good agreement with earlier studies under the same experimental conditions.[6, 18] The N-Domain binding sites show no differences between CaM^{WT} and CaM^{E105A} in terms of binding affinity (Figure 4A and Table 1) and binding cooperativity. This is not surprising considering that E105A mutation is located within the C-domain of the CaM protein. Relative to the C-domain binding sites, CaM^{E105A} shows a ~10-fold lower affinity for Ca^{2+} compared to CaM^{WT} (Table 1). Thermodynamic analysis of the free energy changes upon Ca^{2+} binding to the C-domain binding sites, reveals an interesting picture. The free energy change for CaM^{E105A} is similar to that of CaM^{WT} only when one binding site is occupied (-26.6 kJ/mol and -28.4 kJ/mol respectively), while the total free energy change for binding to both C-domain binding sites is significantly reduced (11.3

kJ/mol less). In addition, for the C-domain of CaM^{E105A}, the free energy gained by pairing of the two binding sites (ΔG_c) is 7.6 kJ/mol, less than that of CaM^{WT}. The aforementioned effects can be interpreted as a significant loss of cooperativity between the two binding sites of this domain, leading to its reduced Ca^{2+}-binding affinity. Molecular modelling (Figure 4B) based on CaM X-ray structure confirms our observations suggesting that glutamic acid (E) at position 105 is directly involved in Ca^{2+}-binding of CaM C-domain and its substitution by an alanine (A) residue dramatically alters the cooperativity between the two binding sites of this domain.

CaM^{E105A} mutant displays reduced RyR2 interaction and defective modulation of [^{3}H]ryanodine binding

It has been demonstrated that RyR2 is the major binding site for CaM along the Z-line in cardiomyocytes and that dissociation of CaM from RyR2 can trigger severe ventricular arrhythmia.[8, 10] Thus, reduced CaM-RyR2 interaction can result in heart failure. To compare the relative RyR2-binding affinities of CaM^{WT} and CaM^{E105A} we used a co-immunoprecipitation assay, as we have previously described.[6, 18] Native RyR2 from pig cardiac SR was immunoprecipitated with a purified anti-RyR2 specific antibody in the presence of either recombinant CaM^{WT} or CaM^{E105A} at different Ca^{2+} concentrations. Association of CaM with RyR2 was analyzed by SDS-PAGE electrophoresis and immunoblot analysis using the anti-CaM monoclonal antibody. Densitometric analysis revealed that RyR2 binding to CaM^{E105A} was dramatically decreased (over ~70%) compared to CaM^{WT} at all Ca^{2+} concentrations (Figure 5A), suggesting that E105A mutation exhibits a major inhibitory effect on the binding of CaM to RyR2.

To further investigate the functional effect of this novel cardiac disease-associated CaM mutation on RyR2 regulation, we performed [^{3}H]ryanodine binding assays.[6, 18] [^{3}H]ryanodine binding assay represents a useful biochemical tool to study the action of different modulators

on the RyR2 function, as it has been demonstrated that the binding of [^{3}H]ryanodine to RyR is dependent upon the functional state of the channel. The effect of CaM^{WT} and its corresponding mutants on [^{3}H]ryanodine binding to RyR2 was examined in a range of different Ca^{2+} concentrations varying from 10nM to 10μM. As shown in Figure 5B, CaM^{WT} significantly reduced the ryanodine binding compared to control (no added CaM protein). In contrast, inhibition of ryanodine binding to RyR2 by CaM^{E105A} mutant was almost abolished at all high Ca^{2+} concentrations, suggesting impaired or no association of RyR2 with this CaM mutant. These findings support our co-immunoprecipitation experiments that indicated a dramatically reduced RyR2- CaM^{E105A} interaction.

Discussion

CaM is one of the most essential cytosolic Ca^{2+} signaling molecules regulating several vital biochemical cascades within eukaryotic cells. CaM is a relatively small protein but displays a high degree of conformational plasticity. Ca^{2+} binding to CaM can induce major conformational changes conferring to CaM the ability to bind to its multiple protein targets (cytosolic or membrane bound) within the cell, regulating their function in a Ca^{2+}-dependent manner.[2] Interestingly, CaM N- and C-lobes display different Ca^{2+}-binding affinities and kinetics upon target binding highlighting the complex mechanism that this versatile molecule translates the Ca^{2+} signals to its several protein targets within the cell.[5, 21]

In cardiac cells, calmodulin is an important regulator of ECC and Ca^{2+} homeostasis through its direct interaction with a number of key proteins, including RyR2, voltage operated potassium, sodium and L-type Ca^{2+} channels.[6] All the three CaM genes (*CALM1, CALM2* and *CALM3*) that encode for an identical protein, expressed in cardiac tissue. Over the last few years, several genetic and clinical reports have identified a number of missense mutations in all three *CALM* genes in individuals with a family history of life-threatening arrhythmogenic

cardiac disorders and early onset sudden cardiac death.[11-16] A recent genetic report identified a novel *de novo* LQTS-associated missense CaM mutation (E105A) in a 6-year-old boy with no family history of heart disease, who experienced an aborted first-episode of cardiac arrest.[17]

In this study, we employed a zebrafish model to investigate for the first time the *in vivo* functional effects of CaM E105A mutation in a vertebrate model system and more specifically to study its impact on the normal zebrafish embryonic heart function. Zebrafish cRNA microinjection experiments revealed that expression of CaM^{WT} and CaM^{E105A} mutant did not have any effect on the normal embryo development as neither gross morphological changes nor difference in the survival rate were observed in comparison to the control group. Interestingly and in contrast with the CaM^{WT}-injected zebrafish, a slight change in the heart morphology was observed in CaM^{E105A}-injected embryos with ~31.5% exhibiting an extended cardiac chamber. Moreover, the expression of CaM^{E105A} mutant in these zebrafish embryos resulted in a specific cardiac phenotype represented in cardiac arrhythmia and an increased heart rate, suggestive to ventricular tachycardia. This observed cardiac phenotype mimicked the human LQTS cardiac phenotype due to abnormal regulation of the ion channels involved in cardiac repolarization. Our molecular modelling, which was based on a CaM X-ray structure, suggested that CaM E105 is directly involved in Ca^{2+}-binding and that its substitution by an alanine could lead to alterations of the Ca^{2+}-binding properties of CaM C-lobe. This was confirmed by our Ca^{2+}-binding studies, which revealed that the C-domain of CaM^{E105A} mutant exhibits a ~10-fold reduced Ca^{2+}-binding affinity compared to CaM^{WT}. This was also in agreement with our thermal denaturation experiments, which showed that in the presence of Ca^{2+}, E105A mutation confers a deleterious impact on the protein stability. RyR2 is the main binding partner for CaM along the Z-line in cardiomyocytes.[8, 22] RyR2 is activated by Ca^{2+} entry into the cytosol through the sarcolemmal voltage-gated channels and this Ca^{2+} entry activates RyR2, triggering RyR2-mediated Ca^{2+} release resulting in a rise of the free cytosolic

Ca^{2+} concentration.[23] CaM binding to RyR2 inhibits Ca^{2+} release both at diastolic and systolic cytosolic Ca^{2+} concentrations.[9] It has been proposed that aberrant CaM dissociation from RyR2 can lead to heart failure.[8] Our biochemical analysis revealed that CaM E105 mutation significantly reduces RyR2-CaM interaction at all Ca^{2+} concentrations, leading to a dramatically reduced modulation of [^{3}H]ryanodine binding to RyR2. These observations could highlight a potential mechanism on how this CaM mutation can lead to arrhythmogenic cardiac disease. A previous study revealed that in zebrafish there are two *ryr2* genes (*ryr2a* and *ryr2b*), which are expressed exclusively in developing central nervous system and cardiac tissue, respectively.[24] It has been previously suggested that in mammals the RyR2 does not contribute to the onset of contractile activity at very early embryonic stages.[24, 25] However, in zebrafish cardiac *ryr* gene (*ryr2b)* is expressed exclusively in the developing heart tissue (precardiac mesoderm) from 14 hpf, 8 hours prior to the onset of cardiac contraction at 22 hpf, and may well contribute to early cardiac development as well as function.[24] It is very intriguing how various pathogenic CaM mutations impose different arrhythmogenic cardiac phenotypes, including CPVT, LQTS and in some cases an overlapping CPVT/LQTS phenotype. This suggests that there might be a mechanistic overlap between CPVT and LQTS caused by CaM mutations.[26] CPVT is characterized by exercise- or stress-induced ventricular arrhythmias, which can lead to syncope or sudden cardiac death. Mutations in RyR2 and other auxiliary proteins, such as calsequestrin-2 and CaM, have been identified in most CPVT-affected patients.[26-30] On the other hand, LQTS can also affect the resting heart and with increasing effect upon adrenergic stimulation.[26, 31] In contrast to CPVT, LQTS is mainly characterized by dysfunction of the sarcolemmal voltage-gated Na^+, Ca^{2+}, and K^+ channels in control of the action potential.[26] Interestingly, many previous studies have proposed different molecular mechanisms and different CaM targets to explain how CaM mutations lead to arrhythmogenic cardiac disease, even within the same arrhythmia type.[6, 15, 20, 23, 26, 32-35] This could be explained

by the multifunctional nature of CaM and the plethora of targets that binds and regulates within the cardiac cells. In a previous study, where we characterized five arrhythmogenic CaM missense mutations we provided biochemical evidence suggesting that one CPVT- (N54I) and two LQTS-associated (D96V and D130G) lead to a defective CaM-RyR2 binding and regulation.[6] Our findings were in overall agreement with the results of another study, which showed that these mutations markedly reduce inhibition of RyR2 Ca^{2+} release during store overload-induced Ca^{2+} release.[26] Moreover, we proposed that another LQTS-associated CaM mutation (F142L) had no apparent effect on RyR2 activity.[6] This again was consistent with another study that showed that F142L CaM mutation does not impair the CaM-dependent inhibition of RyR2.[23] We previously proposed that that the clinical presentation of CPVT or LQTS associated with arrhythmogenic CaM mutations may involve both altered intrinsic Ca^{2+}-binding as well as defective interaction with RyR2.[6] In a similar fashion, Sondergaard et al. also suggested that the regulation of RyR2 Ca^{2+} release is highly sensitive and that aberrant regulation of RyR2 may be a common component of both CPVT and LQTS arrhythmias caused by CaM mutations.[26] It is possible that the LQTS-associated CaM mutations that diminish the CaM-RyR2 interaction; and thus inhibition of the open probability of the channel, may contribute to LQTS phenotypes due to the increased RyR2 Ca^{2+} release. It is also very likely that in some cases the altered intrinsic Ca^{2+}-binding of CaM triggered by the arrhythmogenic mutation, could indeed potentially affect in a higher degree the function of other ion channel complexes in the cardiac cellular arena, and the effect on RyR2 might be more secondary.

In conclusion, taking into consideration the deleterious effects of the several currently identified arrhythmogenic CaM mutations on the normal function of the RyR2 as well as of the other ion channels in cardiac cells, further investigation is required to delineate the molecular mechanisms that CaM mutations lead to life-threatening arrhythmogenic cardiac disease. Establishing a mechanistic link between individual CaM mutants and disease pathogenesis

might provide the clinicians the evidence needed to stratify their patient population and select optimal antiarrhythmic treatment strategies to improve survival of high risk patients. Finally, genetic screening for *CALM* mutations should be recommended and performed in young individuals presented with arrhythmogenic cardiac disorders.

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Author Contributions

M.N. and F.A.L. devised the project strategy. S.I.D., A.T., K.B., G.K.N., B.S.G., E.T., G.N., F.A.L. and M.N. designed/analyzed the experiments, which were performed by S.I.D., A.T., R.S., B.L.C., K.B., I.C., A.S., A.A. and M.N. Finally, M.N. prepared the first manuscript draft, which was revised and approved by all authors.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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Table Legends

Table 1. Dissociation constants and free energy changes of Ca^{2+} binding to C- (ex: 277 nm, em: 320 nm) and N- domain sites (ex: 250 nm, em: 280 nm) at 25 ^{o}C, resolved from fitting of a model-independent two-site Adair function to the experimental data.

Figure Legends

Figure 1. Zebrafish embryos were injected at one cell stage with cRNA encoding CaM^{WT} and CaM^{E105A} mutant. (A) Representative images of 3-day-old zebrafish (left panel) and zebrafish hearts (right panel) corresponding to uninjected, CaM^{WT} and CaM^{E105A} cRNA-injected groups. (B) Heart rate was analyzed using Danioscope software of captured videos for zebrafish larvae at 3 days old. Representative graphs showing the average of heartbeats per minute for uninjected, CaM^{WT} and CaM^{E105A} cRNA-injected groups, respectively. Error bars represent sample standard error of the mean (SEM). (n=18 analyzed per group). **p <= 0.01.

Figure 2. Cardiac activity % was calculated from oscillations in the cardiac signal, with corresponding peaks and lows at regular intervals over time of a total of 6 seconds. Uninjected zebrafish and CaM^{WT} groups exhibited the same pattern of oscillations at approximately 2.5 lows per second, while CaM^{E105A} injection resulted in arrhythmia evident by altered oscillations at a pattern of 2 lows and 3 lows per consecutive two seconds.

Figure 3. (A) Affinity-purified CaM^{WT} (left panel) and CaM^{E105A} (right panel) recombinant proteins (1 μg) were analyzed by 15% SDS-PAGE followed by either Coomassie Brilliant Blue staining or immunoblot analysis using an anti-CaM rabbit monoclonal antibody (1:10,000 dilution). (B) Far-UV CD spectra and thermal stability of CaM^{WT} and CaM^{E105A} mutant. (I) Spectra were recorded at 4 ^{0}C in the presence of 1 mM $CaCl_2$ (CaM^{WT} green; CaM^{E105A} blue) and 1 mM EDTA (CaM^{WT} orange; CaM^{E105A} red). (II) Thermal denaturation curves are shown; lines represent fits assuming a three-state transition. (III) The histogram summarizes melting temperatures (top panel) and van't Hoff's enthalpies (lower panel) resulting from the data shown in (II).

Figure 4. Normalized fluorescence emission intensity profiles of CaM^{WT} (●) and CaM^{E105A} mutant (●) N- (left panel) and C-domain (right panel) as a function of free [Ca^{2+}]. Dashed lines represent the nonlinear least-squares fit of a two-site model-independent Adair function to the collected data. (B) Molecular model of CaM E105A mutation revealing that E105 residue is directly involved in the Ca^{2+} binding of CaM. Overlap of wild type (blue) and CaM E105A (red) energy-minimized models of the EF-hand III binding site with key residues shown as sticks. The model is based on PDB entry 1CLL. Ca^{2+} ions are shown as spheres with their Van-der-Waals radii for emphasis.

Figure 5. (A) Co-immunoprecipitation assays showing the association of CaM^{WT} and CaM^{E105A} mutant with cardiac RyR2. Native RyR2 was immunoprecipitated specifically with Ab^{1093} from CHAPS-solubilized cardiac SR membranes in the presence of 1 μM CaM^{WT} or CaM^{E105A} mutant at different Ca^{2+} concentrations (0, 1 and 10 μM). The presence of RyR2-precipitated CaM proteins was analyzed by 18% SDS-PAGE followed by immunoblot detection analysis using an anti-CaM rabbit monoclonal antibody (1:10,000 dilution). Densitometric analysis and normalization was performed following three independent experiments using three different porcine cardiac SR preparations (histograms). (B) Effect of CaM^{E105A} mutant on [^{3}H]ryanodine binding to cardiac SR vesicles. The binding buffer contained 50 mM HEPES, 25 mM Tris, 500 mM KCl, pH 7.4 with either 1 mM EGTA (<0.01 μM Ca^{2+}) or with the indicated series of free Ca^{2+} concentrations. Normalized [^{3}H]ryanodine binding data are means ± SEM of 4 independent experiments.

Tables

Table 1

		Apparent Dissociation Constant (K_d)	ΔG_1	ΔG_2	ΔG_c
		μM	kJ/mol	kJ/mol	kJ/mol
CaM^{WT}	C-Domain	2.97 ± 0.03	-28.4 ± 0.2	-63.1 ± 0.1	-9.8 ± 0.3
	N-Domain	8.08 ± 0.09	-28.7 ± 0.2	-58.2 ± 0.1	-4.3 ± 0.3
CaM^{E105A}	C-Domain	28.7 ± 0.03	-26.6 ± 0.2	-51.8 ± 0.2	-2.2 ± 0.4
	N-Domain	8.10 ± 0.10	-28.6 ± 0.2	-58.1 ± 0.1	-4.3 ± 0.3

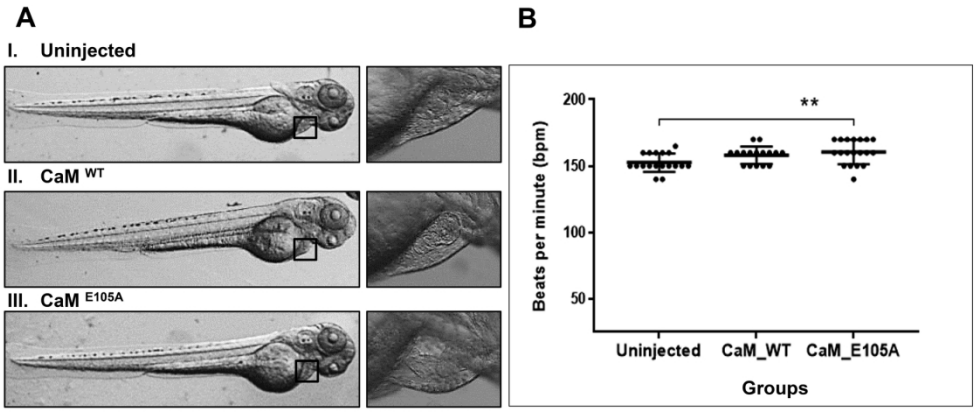

Figure 1

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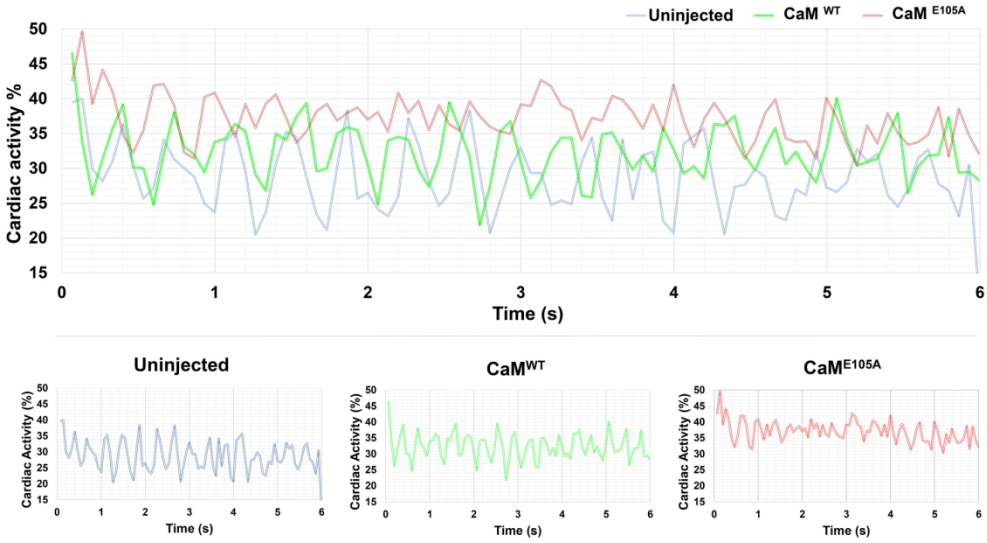

Figure 2

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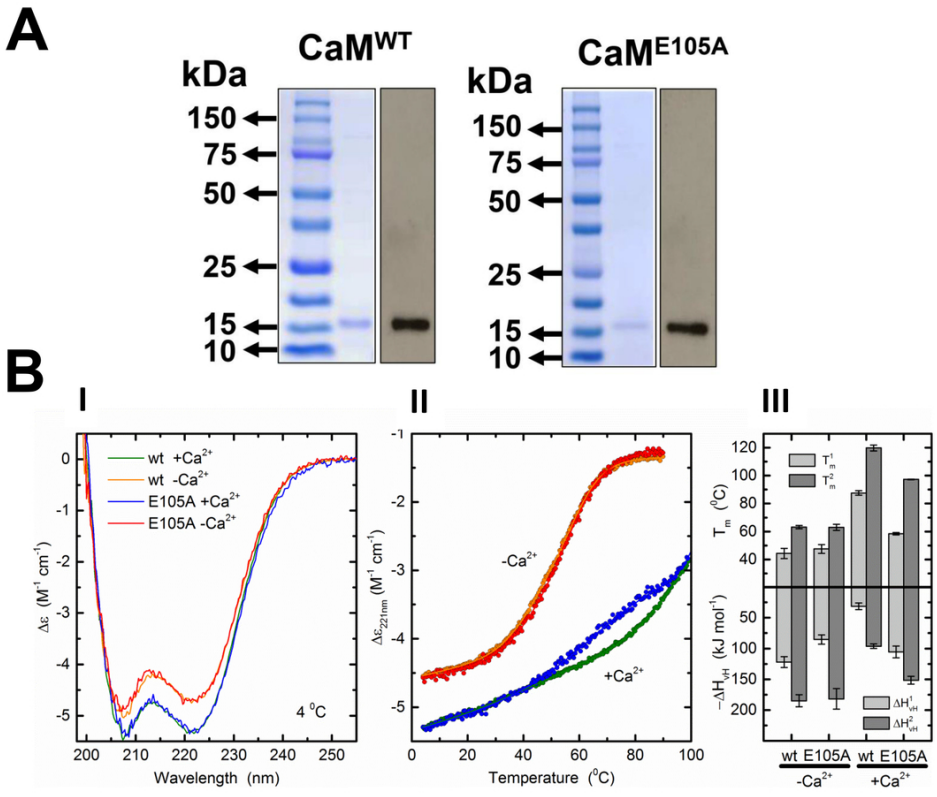

Figure 3

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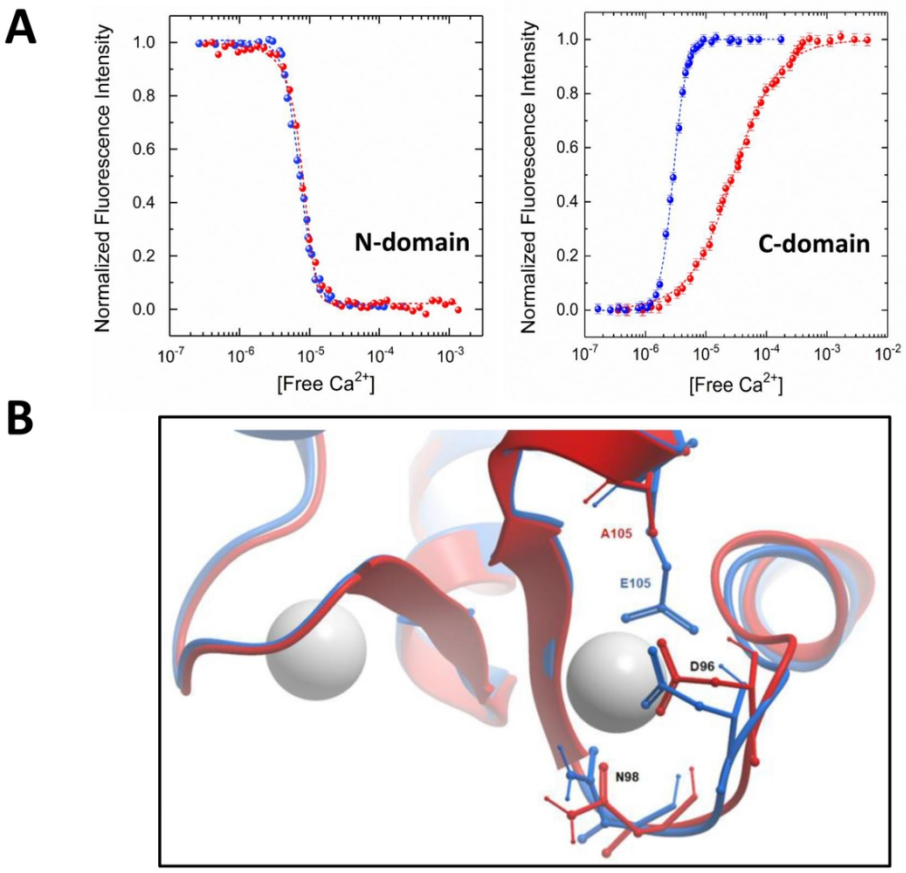

Figure 4

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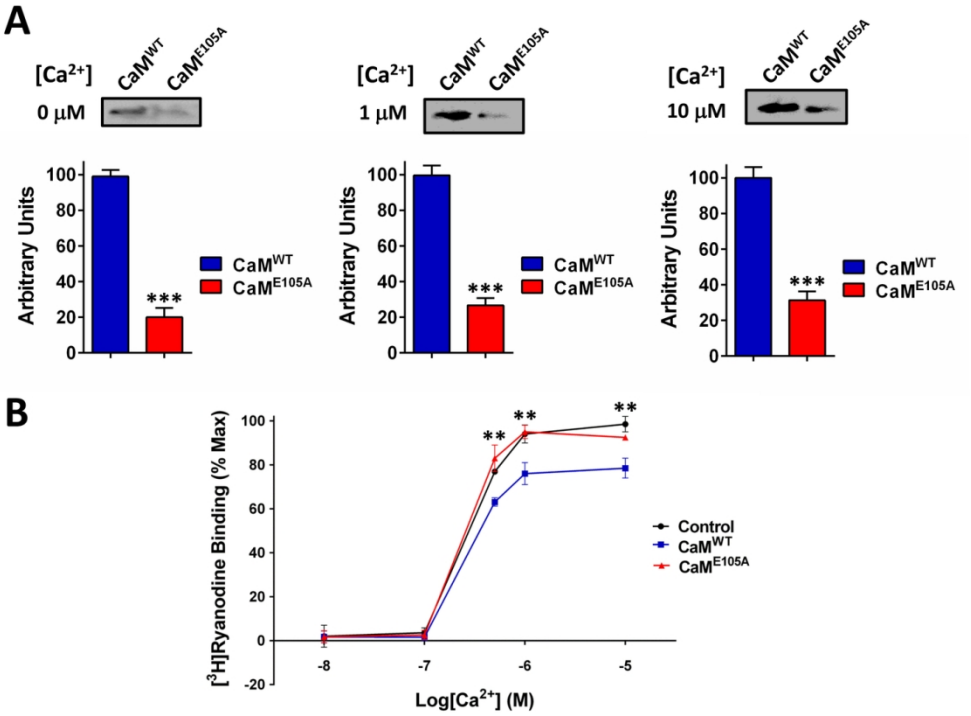

Figure 5

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